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(54) Title: IMMUNITY ENHANCING LACTIC ACID BACTERIA

(57) Abstract

Novel bacteria *Lactobacillus rhamnosus* HN001 and HN 067, *Lactobacillus acidophilus* HN017, and *Bifidobacterium lactis* HN019 are claimed. Each strain provides immune enhancing effects when ingested.

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IMMUNITY ENHANCING LACTIC ACID BACTERIA

Technical Field

This invention relates to novel strains of lactic acid bacteria and their use in enhancing immunity.

Background Art

The consumption of products containing lactic acid bacteria (LAB) is associated with a range of health benefits including enhancement of immunity. There are thousands of strains of lactic acid bacteria but only some strains exhibit health-promoting properties. The ability of these bacteria to tolerate acids and bile salts, adhere to mucosal epithelial cells, and to survive passage through the gastrointestinal tract is considered an important criterion for selection of health-promoting strains. Only a few strains of lactic acid bacteria with proven health benefits have been identified to date.

Strains of LAB showing good adhesion to the cells of the mucosal epithelium of the small intestine thereby lending themselves to therapeutic applications are known from New Zealand Patent 248057. The micro-organisms described in this patent enhance both natural immunity (phagocyte function) and acquired immunity (antibody responses and lymphocyte proliferation responses).

It is desirable to have other LAB bacteria that enhance a broad spectrum of immune responses including phagocyte function.

It is an object of this invention to go some way towards achieving these desiderata or at least to offer the public a useful choice of immune enhancing lactic acid bacteria.

Disclosure of the Invention

Accordingly, in one aspect the invention may be said broadly to consist of a biologically pure culture of *Lactobacillus rhamnosus* HN001, AGAL deposit number NM97/09514 dated 18 August 1997.

In another aspect the invention may be said broadly to consist of a biologically pure culture of *Lactobacillus rhamnosus* HN067, AGAL deposit number NM97/01925 dated 17 February 1998.

In another aspect the invention may be said broadly to consist of a composition of a biologically pure culture of any one of *Lactobacillus acidophilus* HN017, AGAL deposit number NM97/09515 dated 18 August 1997, *Lactobacillus rhamnosus* HN001, *Lactobacillus rhamnosus* HN067 or *Bifidobacterium lactis* HN019, AGAL deposit number NM97/09513 dated 18 August 1997 in an immunostimulating concentration, with a physiologically acceptable excipient or diluent.

In one embodiment said composition contains any two or more of said strains.

Preferably said physiologically acceptable excipient or diluent is a food.

Preferably said food is any one of cultured milk, yoghurt, cheese, milk drink or milk powder.

Alternatively said composition is a pharmaceutical composition and said excipient or diluent is pharmacologically acceptable excipient or diluent.

Immunity enhancing, physiologically acceptable, biologically pure strains of homologues or mutants of any one of the strains:

Lactobacillus acidophilus HN017,
Lactobacillus rhamnosus HN001,
Bifidobacterium lactis HN019, or
Lactobacillus acidophilus HN067.

In another embodiment the invention may be said broadly to consist of a method of enhancing natural and acquired immunity which comprises administering to a mammal any one of the above biologically pure cultures at an immunostimulating dosage rate.

In another embodiment substantially biologically pure cultures of two or three of the above-defined strains are present.

Preferably said culture is administered in the form of a composition with a physiologically acceptable excipient or diluent.

Preferably said physiologically acceptable excipient or diluent is a food.

Preferably said food is cultured milk, yoghurt, cheese, milk drink or milk powder.

This invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively, and any or all combinations of any two or more of said parts, elements or features, and where specific integers are mentioned herein which have known equivalents in the art to which this invention relates, such known equivalents are deemed to be incorporated herein as if individually set forth.

Brief Description of the Drawings

Figure 1 shows the effect of supplementation of mice with product fermented with *L. rhamnosus* HN001 or unfermented product containing *L. rhamnosus* HN001 on phagocyte activity of peripheral blood leukocytes as described in example 5. BALB/c mice were fed on milk based diets containing 10^9 cfu (per day) *L. rhamnosus* HN001 in either fermented or unfermented product for 14 days. Phagocytic activity of peripheral blood leukocytes was determined using flow cytometry and fluoroscein isothiocyanate-labelled *Escherichia coli*. Values are mean ± standard error. Significant differences (ANOVA, the SAS program) from the control: **P<0.0001.

Figure 2 shows the effect of supplementation of mice with live *L. rhamnosus* HN001 or heat killed *L. rhamnosus* HN001 on phagocytic activity of peripheral blood leukocytes as described in example 7. BALB/c mice were fed on milk based diets and

orally administered 10^9 cfu (per day) of either live or heat killed *L. rhamnosus* HN001 for 14 days. Phagocytic activity of peripheral blood leukocytes and peritoneal macrophages were determined using flow cytometry and fluoroscein isothiocyanate - labelled *Escherichia coli.* Values are mean ± standard error. Significant differences (ANOVA, the SAS program) from the control, **P<0.0001.

Figure 3 shows the effect of supplementation of mice with *L. rhamnosus* HN001 or *B. lactis* HN019 on bacteria translocation in mice challenged with *S. typhimurium* as described in example 8. Unsupplemented and *B. lactis* HN019, or *L. rhamnosus* HN001 supplemented BALB/c mice were orally challenged with *S. typhimurium* following continuous daily supplementation. Six days after challenge mice were humanely killed and their livers and spleens were harvested for monitoring bacterial translocation. Tissue suspensions from the harvested organs were then cultured on MacConkey agar plates for 24-48hr prior to enumeration. Values are mean ± standard error. Significant differences (ANOVA, the SAS program) from the control: *P<0.05.

Figure 4 shows the effect of supplementation of mice with *L. rhamnosus* HN001 or *B. lactis* HN019 on the phagocytic activity of peripheral blood leukocytes from mice challenged with *S. typhimurium* as described in example 8. Unsupplemented and *B. lactis* HN019, or *L. rhamnosus* HN001 supplemented BALB/c mice were orally challenged with *S. typhimurium* following continuous daily supplementation. Phagocytic activity of peripheral blood leukocytes was determined six days after challenge using flow cytometry and fluoroscein isothiocyanate-labelled *Escherichia coli.* Values are mean ± standard error. Values (mean ± standard error) with different superscripts are significantly different (ANOVA, the SAS program): P<0.01.

Figure 5 shows the effect of supplementation of mice with *L. rhamnosus* HN001 or *B. lactis* HN019 on the proliferative responses of spleen lymphocytes from mice challenged with *S. typhimurium* as described in example 8. Unsupplemented and *B. lactis* HN019, or *L. rhamnosus* HN001 supplemented BALB/c mice were orally challenged with *S. typhimurium* following continuous daily supplementation. Six days after challenge the proliferative responses of spleen lymphocytes were measured colourimetrically following the incorporation of 5-bromo-2'-deoxyuridine for the final 16hrs of the 96hr incubation. Values (mean ± standard error) with different superscripts are significantly different (ANOVA, the SAS program): P<0.01).

Modes of Carrying out the Invention

Freeze dried cultures of the four bacterial strains have been deposited at the Australian Government Analytical Laboratories (AGAL), The New South Wales Regional Laboratory, 1 Suakin Street, Pymble, NSW 2073, Australia. Details of the deposits are:

Strain	Number	Date
L. acidophilus HN017	NM97/09515	18 August 1997
L. rhamnosus HN001	NM97/09514	18 August 1997
B. lactis HN019	NM97/09513	18 August 1997
L. rhamnosus HN067	NM97/01925	11 February 1998

The four strains identified above have been found to enhance a broad spectrum of immune responses including both natural and acquired immune responses.

Example 1 - Morphology and General Properties

RAPD analysis, 16S rRNA sequencing and SDS-PAGE analyses were used to confirm taxonomical characterisation of strains. It was also found that *L. acidophilus* HN017 was genetically different from *L. acidophilus* (LC1) of New Zealand Patent No. 248057.

RAPD analysis, 16S rRNA sequencing and SDS-PAGE analyses were used to confirm taxonomical characterisation of *L. rhamnosus* HN067; species-specific primers used for characterisation of *L. rhamnosus* HN067 at molecular level included Pr I (forward) 5-CAGACTGAAAGTCTGACGG-3 and Pha II (reverse) 5-GCGATGCGAATTTCTATTATT-3.

The morphology and sugar fermentation properties of this strain are detailed in Tables 1 and 2.

Table 1 - Morphology and other characteristics

L. acidophilus HN017	*L. rhamnosus* HN001	*B. lactis* HN019	*L. rhamnosus* HN067
Short to medium rods with rounded ends, generally occurring singly or in pairs or short chains, when grown in MRS broth. Gram positive, non-spore forming, catalase negative facultatively anaerobic rods with optimum growth temperature of 37±1°C and optimum pH of 6.0 - 6.5. These are obligately homofermentative bacteria and no gas is produced from glucose.	Short to medium rods with square ends in chains, generally 0.7 x 1.1 x 2.0 - 4.0 µm, when grown in MRS broth. Gram positive, non-mobile, non-spore forming, catalase negative facultative anaerobic rods with optimum growth temperature of 37±1°C and optimum pH of 6.0 - 6.5. These are facultatively heterofermentative bacteria and no gas produced from glucose.	Microaerophilic to anaerobic rods with characteristic shapes such as middle enlarged cells, 'V' or palisade arrangement of cells when grown on TPY agar slabs. In MR5 broth with 0.05% cysteine hydrochloride, they form middle-enlarged cells and club shaped (spatulated extremities) cells. Gram positive, non-motile and non-spore forming, catalase negative rods with optimum growth temperature of 37±1°C and optimum pH of 6.0 - 7.0. Fructose-6-phosphate phospho-ketolase positive.	Short to medium rods with square ends in chains, generally 0.7 x 1.1 x 2.0 to 4.0 µm, when grown in MRS broth. Gram positive, catalase negative, non-mobile, non spore-forming, facultative anaerobic rods with optimum growth temperature of 37±1°C and optimum pH of 6.0 to 6.5. These are facultatively heterofermentative bacteria and no gas produced from glucose.

Table 2 - Carbohydrate fermentation pattern of selected Lactobacillus and Bifidobacterium strains

Sl. No.	Name of the bacterium	Score*
1	*L. acidophilus* HN 017	5755546
2	*L. rhamnosus* HN001	5757177
3	*B. lactis* HN019	1051622
4	*L. rhamnosus* HN067	5757175

API 50 CH sugar fermentation kit was used to determine the sugar fermentation pattern.

* The scores are based on scores of 22 prominent sugars (Bergey's manual)

Example 2 - Adhesion to Intestinal Cells

The ability of probiotic strains to adhere to human intestinal epithelial cells (HT-29 and CaCo-2) was assessed *in vitro* using differentiated cell-lines. Monolayers of HT-29 and CaCo-2 cells were grown on cover slips and placed in multi-well dishes. 10^8 cfu/ml of LAB in 1 ml of spent culture supernatant was then added to cell layers along with 1 ml of DMEM medium and incubated for 1 hr at 37°C in 10% CO_2-90% air. Monolayers were washed 4 times with PBS, fixed in methanol, Gram strained and the number of bacteria adhering to epithelial cells determined microscopically. On average, 20 fields were counted and the results are summarised in Table 3.

Table 3 - Adherence to HT-29 and CaCo-2 cell lines*

STRAIN	HT-29	CaCo-2
L. acidophilus HN 017	98 ± 17	171 ±16
L. rhamnosus HN 001	161 ± 18	218 ± 35
B. lactis HN 019	188 ± 27	194 ± 25

* Number (mean ± SEM) of bacteria/100 epithelial cells

Example 3 - Enhancement of Natural and Acquired Immunity

The immunoenhancing effects of the three strains *L. rhamnosus* HN001, *L. acidophilus* HN017 and *B. lactis* HN019 were examined by determining phagocyte (blood leukocytes and peritoneal macrophage) function, and quantifying concentrations of specific antibodies to protein antigens used for mimicking responses to vaccines in mice.

The following experimental protocol was used:

1. Six-to-seven week old BALB/c mice, weighing 20-30g were used.
2. Mice were randomly allocated to different treatment groups (Table 4)
3. Mice were fed *L. acidophilus* HN017, *L. rhamnosus* HN001 or *B. lactis* HN019 (10^9 cfu/day) in 50µl skim milk for 10 days. Control mice received 50µl of skim milk powder only.
4. All mice received skim milk powder based diet throughout the experiment.

Blood leukocytes and macrophages from mice receiving *L. acidophilus* HN017, *L. rhamnosus* HN001 or *B. lactis* HN019 showed significantly greater phagocytic capacity compared with cells from control mice (Table 4). The production of oxygen radicals (oxidative burst) by leukocytes from probiotic fed mice was also higher than the control mice (data not shown).

Table 4 - The effect of dietary *L. acidophilus* HN017, *L. rhamnosus* HN001 and *B. lactis* HN019 on phagocyte function in mice

Treatment	% Blood leukocytes with phagocytic activity	% Peritoneal macrophage with phagocyte activity
Control	14.33 ± 0.87	66.1 ± 3.5
L. acidophilus HN017	22.7 ± 1.21**	79.0 ± 1.0 **
L. rhamnosus HN001	24.84 ± 0.93 **	80.5 ± 1.8 **
B. lactis HN019	23.19 ± 0.95 **	77.4 ± 2.6 *

BALB/c mice were orally administered with 10^9 cfu (per day) *L. acidophilus* HN017, *L. rhamnosus* HN001 or *B. lactis* HN019 for 10 days. Phagocytic activity of blood leukocytes and peritoneal macrophages was determined using flow cytometry and fluorescein isothiocyanate - labelled *Escherichia coli.* Values are mean ± standard error. Significant differences (Students t test) from the control: *P < 0.05, **P <0.01.

The concentration of specific IgG antibodies in the sera and in the intestinal washings of mice receiving *L. acidophilus* HN017, *L. rhamnosus* HN001 or *B. lactis* HN019 was also greater than those of control mice (Table 5).

Table 5 - The effect of dietary *L. acidophilus* HN017, *L. rhamnosus* HN001 and *B. lactis* HN019 on serum and mucosal antibody responses

Treatment	Serum antibody response (units/ml)	Mucosal antibody response (units/ml)
Control	80.2 ± 6.0	1350 ± 96.0
L. acidophilus HN017	134.6 ± 25.2 *	1548 ± 270.0
L. rhamnosus HN001	118.5 ± 12.5 **	1512 ± 198.0
B. lactis HN019	158.1 ± 51.6 ***	1548 ± 234.0

BALB/c mice were orally administered with 10^9 cfu (per day) *L. acidophilus* HN017, *L. rhamnosus* HN001 or *B. lactis* HN019 for 10 days. Mice were immunised with cholera toxin (an antigen used to mimic enteric infection) on days 0 and 7. The concentration of specific antibodies in serum and intestinal secretions were measured using ELISA on day 10. Values represent mean ± standard error. Significant differences (Students t test) from control: *P = 0.08; **P<0.05; ***P<0.01.

Example 4 - Immunostimulating Effects Following Supplementation with LAB for Four Weeks

The immunostimulating effects of *L. acidophilus* HN017, *L. rhamnosus* HN001, and *B. lactis* HN019 were assessed in mice using the following experimental protocol:

1. Six-to-seven week old BALB/c mice, weighing 20-30g were used.
2. Mice were randomly allocated (18/group) to different treatment groups.
3. After acclimatisation (for 7 days), mice were given 10^9 cfu (per day) *L. acidophilus* HN017, *L. rhamnosus* HN001, or *B. lactis* HN019, in 50 µl skim milk, for 28 days (from day 0 to day 28). Control mice received 50 µl skim milk (without any micro-organisms) only.
4. Mice were offered a skim milk powder based-diet and water *ad libitum*, throughout the experiment.
5. Immunostimulating effects were assessed by monitoring phagocytic activity of blood leukocytes and peritoneal macrophages, NK-cell activity of splenic lymphocytes, lymphocyte proliferation (spleen cells) responses to a T-cell mitogen, ConA (an indicator of cell-mediated immunity) and antibody responses to Tetanus vaccine.

As seen in Table 6, leukocytes (neutrophils, monocytes and macrophages) from mice receiving *L. acidophilus* HN017, *L. rhamnosus* HN001, or *B. lactis* HN019 exhibited significantly greater phagocytic activity (an indicator of natural immunity) than leukocytes from control mice.

Table 6 - The effect of dietary *L. acidophilus* HN017, *L. rhamnosus* HN001, and *B. lactis* HN019 in mice

Treatment	% Blood leukocytes with phagocytic activity	% Peritoneal macrophages with phagocytic activity
Control	15.5	72.67
L. acidophilus HN017	29.4**	82.2*
L. rhamnosus HN001	24.2**	82.8**
B. lactis HN019	31.1**	83.0**

Mice (18/group) were given 10^9 cfu (per day) *L. acidophilus* HN017, *L. rhamnosus* HN001, or *B. lactis* HN019 in 50 µl skim milk for 28 days. Phagocytic activity of blood leukocytes/peritoneal macrophages was determined on day 28 using flow cytometry and fluorescein isothiocyanate-labelled *E. coli*. Values are least square means. Significant differences (the SAS analysis): *P<0.002, **P<0.0005.

Consumption of *L. acidophilus* HN017, *L. rhamnosus* HN001, or *B. lactis* HN019 for 28 days also resulted in an increase in the NK-cell activity, lymphocyte proliferation responses to ConA and antibody responses to Tetanus vaccine. For all these indicators of immunocompetence, mice receiving *L. acidophilus* HN017, *L. rhamnosus* HN001, or *B. lactis* HN019 had higher responses than those of control mice (Table 7).

Together these results show that supplementation for extended periods with *L. acidophilus* HN017, *L. rhamnosus* HN001, or *B. lactis* HN019 is able to induce a sustained enhancement in several aspects of natural and acquired immunity.

Table 7 - The effect of dietary *L. acidophilus* HN017, *L. rhamnosus* HN001, and *B. lactis* HN019 on NK cell activity and lymphocyte proliferation responses to ConA and antibody responses to Tetanus vaccine.

ConA Treatment	NK cell activity (%)	Lymphocyte proliferation to ConA (absorbance)	Antibody responses to Tetanus vaccine (units/ml)
Control	8.8	1.4 ± 0.125	402.5 ± 41.4
L. acidophilus HN017	9.9	1.6 ± 0.44	923.9 ± 116.0*
L. rhamnosus HN001	11.5	1.8 ± 0.1*	711.5 ± 127.2*
B. lactis HN019	10.5	1.7 ± 0.5*	844.6 ± 134.7*

Mice (18/group) were given 10^9 cfu (per day) *L. acidophilus* HN017, *L. rhamnosus* HN001, or *B. lactis* HN019 in 50 µl skim milk for 28 days (i.e. from days 0 to 28). NK-cell activity of splenic lymphocytes was determined on day 28 using flow cytometry and D275-labelled Yac-1 cells. Lymphocyte proliferation responses of splenic lymphocytes to ConA were assessed on day 28 using a commercial cell

proliferation kit (Boehringer Mannheim, Germany). For antibody responses, mice were immunised with Tetanus vaccine (50 µl/dose, CSL, Australia) on days 7 and 21. The concentration of specific antibodies were determined using an ELISA; antigen supplied by the vaccine manufacturers (CSL, Australia) was used for coating plates. Values are least square means of 18 mice. Significant differences (the SAS analysis): *P <0.05.

Example 5 - Enhancement of Natural and Acquired Immunity Using Fermented versus Unfermented Products

The aim was to assess the immunoenhancing efficacy of yoghurt made (fermented) using the probiotic strain *L. rhamnosus* HN001 compared to unfermented product containing *L. rhamnosus* HN001. The immunoenhancing effects were examined by determining the phagocyte function (peripheral blood leukocytes and peritoneal macrophages) and lymphocyte proliferative responses to a B-cell mitogen (LPS).

The following experimental protocol was used:

1. Six-to-seven week old BALB/c mice, weighing 20-30g were used.
2. Mice were randomly allocated to different treatment groups.
3. Control mice received a whole milk powder-based diet throughout the experiment.
4. Test mice received 2.5g yoghurt made using *L. rhamnosus* HN001 (10^9 cfu/day) or 2.5g whole milk containing *L. rhamnosus* HN001 (10^9 cfu/day) per day as well as a whole milk powder based diet for 14 days.

Results

Mice receiving yoghurt made with *L. rhamnosus* HN001 or whole milk containing *L. rhamnosus* HN001 displayed a significantly higher level of phagocytic activity of peripheral blood leukocytes than was observed in mice receiving the control diet (Fig

1). This increase was seen irrespective of whether the *L. rhamnosus* HN001 was delivered in the yoghurt (fermented with *L. rhamnosus* HN001) or unfermented product containing *L. rhamnosus* HN001. There was no difference in the level of phagocytic activity between mice receiving the fermented yoghurt made using *L. rhamnosus* (HN001) compared to unfermented WMP product containing *L. rhamnosus* (HN001).

Both the unfermented and *L. rhamnosus* HN001 fermented product fed mice showed higher lymphocyte proliferative responses to LPS than the control mice (Table 8). There was no significant difference in the response between mice receiving unfermented product containing *L. rhamnosus* HN001 and mice receiving product fermented with *L. rhamnosus* HN001.

Table 8 - The effect of fermented and unfermented *L. rhamnosus* HN001 on lymphocyte proliferative responses in mice

Treatment	Lymphocyte proliferation to LPS (absorbance)
Control (WMP)	0.4699 ± 0.028
WMP Fermented with *L. rhamnosus* HN001	0.5361 ± 0.028
Unfermented WMP with *L. rhamnosus* HN001	0.5518 ± 0.028*

BALB/c mice were fed on milk based diets containing 10^9 cfu (per day) *L. rhamnosus* HN001 in either unfermented product or yoghurt made with *L. rhamnosus* HN001 (fermented product) for 14 days. Control mice received milk-based diet without any LAB. Proliferative responses were measured colourimetrically following the incorporation of 5-bromo-2'-deoxyuridine for the final 16hrs of the 96hr incubation. Values are means ± standard error. Significant differences (Students t test) from the control: *P=0.05.

Together these results suggest that supplementation with *L. rhamnosus* HN001 enhances a range of immune functions including phagocytic activity and lymphocyte

cell proliferation. *L. rhamnosus* HN001 presented in either fermented or unfermented product is effective at eliciting enhancement of immune function, with fermented product giving a greater response for some functions and unfermented being superior in others.

Example 6 - Enhancement of Natural and Acquired Immunity by *L. rhamnosus* HN067

Experiment 1.

The immunoenhancing effects of *L. rhamnosus* HN067 were examined by monitoring phagocytic capacity of peripheral blood leukocytes and peritoneal macrophages (indicator of non-specific immunity), and quantifying concentrations of specific antibodies to an immunisation antigen, cholera toxin (used for mimicking responses to enteric vaccines) in mice.

The following experimental protocol was used:

1. Six-to-seven week old BALB/c mice, weighing 20-30g were used. They were fed on a skim milk-based diet throughout the experiment.
2. Mice in the test group (n=6) were orally administered *L. rhamnosus* HN067 (10^9 cfu/day) in 50µl skim milk for 10 days. Control mice (n=6) received 50µl of skim milk powder (without any LAB) only.

Results

Blood leukocytes and peritoneal macrophages from mice receiving *L. rhamnosus* HN067 showed significantly greater phagocytic activity (enhanced phagocyte function) compared with cells from control mice. The results are set out in Table 9 below.

Table 9 - The effect of dietary *L. rhamnosus* HN067 on phagocyte function

Treatment	% Blood leukocytes with phagocytic activity	% Peritoneal macrophages with phagocytic activity
Control	13.1 ± 1.5	76.4 ± 1.9
L. rhamnosus HN067	23.7 ± 1.5**	87.2 ± 1.9*

BALB/c mice (6/group) were fed on milk-based diet with or without oral administration of *L. rhamnosus* HN067 (10^9 cfu/day) for 10 days. Phagocytic activity of blood leukocytes and peritoneal macrophages were determined using flow cytometry and fluorescein isothiocyanate - labelled *E. coli*. Values represent least square mean ± standard error LSM. Significant differences (the SAS program) from the control: *P=0.0005, **P=0.0001.

The concentration of specific antibodies to cholera toxin, an antigen used for oral immunisation, in the sera and in the intestinal washings of mice receiving *L. rhamnosus* HN067 was also significantly greater than those of control mice (Table 10).

Table 10 - The effect of dietary supplementation with *L. rhamnosus* HN067 on serum and mucosal antibody responses to cholera toxin

Treatment	Serum antibody response (units/ml)	Mucosal antibody response (units/ml)
Control	63.1 ± 43.2	1969.7 ± 279.5
L. rhamnosus HN067	246.5 ± 43.2**	2995.5 ± 465.2*

BALB/c mice were fed on milk-based diet with or without *L. rhamnosus* HN067 (10^9 cfu/day) for 10 days. Mice were immunised orally with cholera toxin (10µg/dose), an antigen used to mimic enteric infection, on days 0 and 7. Antibody levels in serum and intestinal secretions were measured using ELISA on day 10. Values represent

least square mean ± standard error LSM. Significant differences (the SAS program) from control: *P=0.02; **P=0.0039.

Experiment 2.

The immunostimulating effects of *L. rhamnosus* HN067 were assessed in mice using the following experimental protocol:

1. Six-to-seven week old BALB/c mice, weighing 20-30g were used. They were offered skim milk powder based diet and water *ad libitum*, throughout the experiment.
2. After acclimatisation for 7 days, mice in group 1 (n=20) were orally administered with 10^9 cfu (per day) *L. rhamnosus* (HN067) in 50 µl skim milk (group 1 n = 20) for 14 days. Control mice (group 2, n = 20) received skim milk without any microorganisms.
3. Immunostimulating effects were assessed by monitoring phagocytic activity of blood leukocytes and peritoneal macrophages, and spleen lymphocyte proliferation responses to phytohaemagglutinin (PHA) and lipopolysaccharide (LPS) (T and B-cell mitogens respectively).

Results

Blood leukocytes and peritoneal macrophages from mice receiving *L. rhamnosus* HN067 exhibited significantly greater phagocytic activity (an indicator of natural immunity) than leukocytes and macrophages from control mice (Table 11).

Table 11 - The effect of dietary *L. rhamnosus* HN067 on phagocyte function in mice

Treatment	% Blood Leukocytes with phagocytic activity	% Peritoneal macrophages with phagocytic activity
Control	13.7 ± 0.07	64.6 ± 2.1
L. rhamnosus HN067	22.5 ± 0.07**	75.8 ± 1.7*

BALB/c mice were fed on milk-based diet with or without oral administration of *L. rhamnosus* HN067 (10^9 cfu/day) for 14 days. Phagocytic activity of blood leukocytes/peritoneal macrophages were determined on day 14 using flow cytometry and fluorescein isothiocyanate-labelled *E. coli*. Values represent least square mean ± standard error LSM. Significant differences (the SAS program): *P=0.002, **P=0.0001.

Mice receiving *L. rhamnosus* HN067 for 14 days also displayed higher lymphocyte proliferation responses to PHA and LPS compared with control mice (Table 12).

Table 12 - The effect of *L. rhamnosus* HN067 supplementation on lymphocyte proliferation responses to PHA and LPS

ConA Treatment	Lymphocyte proliferation to PHA	Lymphocyte proliferation to LPS
Control	1.18 ± 0.08	0.99 ± 0.07
L. rhamnosus HN067	1.37 ± 0.07*	1.24 ± 0.06**

BALB/c mice were fed on milk-based diet with or without oral administration of *L. rhamnosus* HN067 (10^9 cfu/day) for 14 days. Lymphocyte proliferation responses of spleen cells to PHA and LPS were assessed on day 14 using a commercial cell proliferation kit (Boehringer Mannheim, Germany). Values represent least square mean ± standard error LSM. Significant differences (the SAS program): *P<0.08, **P<0.01.

In summary, mice receiving *L. rhamnosus* HN067 displayed significant enhancement of a range of host immune responses including leukocyte phagocytic function, antibody responses to oral immunisation, and lymphocyte proliferation responses to T and B-cell mitogens. Blood leukocytes (neutrophils and monocytes) and macrophages are major effectors of natural immunity and play a major role in protection against microbial infections. A correlation between *in vitro* lymphocyte proliferation responses to mitogens (T- and B-cell mitogens) and immunocompetence of an individual is also well documented. Therefore, these results suggest that

supplementation with *L. rhamnosus* HN067 is able to enhance several aspects of natural and acquired immunity.

Example 7 - Enhancement of Natural and Acquired Immunity Using Live and Heat Killed *L. rhamnosus* HN001

The aim of the present study was to investigate the immunoenhancing effects of the probiotic strain *L. rhamnosus* HN001 when presented in either the live or heat killed form. The effect on immune function was assessed by determining phagocytic activity of peripheral blood leukocytes. The effect of live and heat killed *L. rhamnosus* HN001 on humoral immunity was investigated by immunising mice with cholera toxin, and measuring the concentrations of specific antibodies produced.

The following experimental protocol was used:

1. Six-to-seven week old BALB/c mice, weighing 20-30g were used.
2. Mice were randomly allocated to different treatment groups.
3. Control mice received a skim milk powder based diet throughout the experiment.
4. Test mice receive either 10^9 cfu/day of live *L. rhamnosus* HN001 or 10^9 cfu/day heat killed *L. rhamnosus* HN001 per day as well as a skim milk powder-based diet for 14 days.
5. Mice were orally immunised with cholera toxin on day 0 and day 7 of feeding.

Results

L. rhamnosus HN001 feeding significantly enhanced the level of phagocytic activity of peripheral blood leukocytes compared to mice receiving the control diet (Fig 2). This increase was seen irrespective of whether the *L. rhamnosus* HN001 was delivered in the live or heat killed form. There was no difference in the level of phagocytic activity between the mice receiving live *L. rhamnosus* HN001 compared to heat killed *L. rhamnosus* HN001.

Feeding of both live and dead *L. rhamnosus* HN001 induced an increase in both serum and mucosal antibody responses compared to the control mice. However, the

level of response was significantly greater in the mice fed the live *L. rhamnosus* HN001 (Table 13).

Table 13 - The effect of live and heat killed *L. rhamnosus* HN001 on serum and mucosal antibody responses to Cholera Toxin in mice

Treatment	Serum antibody response (units/ml)	Mucosal antibody response (units/ml)
Control	88.69 ± 18.52	708.6 ± 146.9
Live *L. rhamnosus* HN001	214.89 ± 62.33*	2054.5 ± 285.8***
Heat Killed *L. rhamnosus* HN001	174.89 ± 44.78	1533.6 ± 319.3

BALB/c mice were fed on milk-based diets and orally administered 10^9 cfu (per day) *L. rhamnosus* HN001 in either live or heat killed form for 14 days. Control mice received no LAB. Mice were orally immunised with Cholera Toxin on days 0 and 7. Antibody responses (serum and intestinal secretions) were measured using an ELISA on day 14. Values are mean ± standard error. Significant differences (Students t test) from the control: *P=0.05, ***P=0.0005.

These results suggest that both live and heat killed *L. rhamnosus* HN001 are able to enhance aspects of natural and acquired immunity in mice.

Example 8 - Anti-infection properties of *B. lactis* HN019 and *L. rhamnosus* HN001

The aims of the current study were to:

1. Assess the protection efficacy of *B. lactis* HN019 and *L. rhamnosus* HN001 against the gastrointestinal pathogen *Salmonella typhimurium*.
2. Determine the role of immunostimulation induced by *B. lactis* HN019 and *L. rhamnosus* HN001 in protection against *S. typhimurium* infection in mice.

Anti-infection properties were assessed by measurement of bacterial translocation to the liver and spleen. The immunoenhancing effects were examined by determining the phagocyte function (peripheral blood leukocytes and peritoneal macrophages) and lymphocyte proliferative responses to a T-cell mitogen (PHA).

The following experimental protocol was used:

1. Six-to-seven week old BALB/c mice, weighing 20-30g were used.
2. Mice were randomly allocated to 4 difference treatment groups and were individually housed.
3. All mice received a skim milk powder based diet throughout the experiment.
4. Test mice commenced daily feeding of *B. lactis* HN019 or *L. rhamnosus* HN001 (10^9 cfu/day) 7 days prior to challenge, and continued for the duration of the trial.
5. Mice administered with *B. lactis* HN019 or *L. rhamnosus* HN001 and a control group (no LAB) were orally challenged with *Salmonella typhimurium* (ATCC 1772) 8×10^5 cfu/day for 5 days starting on day 7.
6. An uninfected control group did not receive *S. typhimurium* challenge.
7. On day 6 after challenge mice were used for the measurement of bacterial translocation to the liver and spleen, and for immune function assessment.

Results

Both the *B. lactis* HN019 and *L. rhamnosus* HN001 supplemented mice showed significantly lower levels of bacterial translocation into the liver and spleen than the *S. typhimurium* alone fed mice (Fig 3).

Challenge infection resulted in a significant suppression of phagocyte function (Fig 4); the phagocytic activity of control mice challenged with *S. typhimurium* was significantly lower than that of the uninfected mice. However, infection with *S. typhimurium* had no effect on the phagocytic ability of peripheral blood leukocytes of mice supplemented with *B. lactis* HN019 or *L. rhamnosus* HN001. This was shown by similar levels of phagocytic activity in mice supplemented with *B. lactis*

HN019 or *L. rhamnosus* HN001 and challenged with *S. typhimurium* and the normal uninfected control mice.

Both the *B. lactis* HN019 and *L. rhamnosus* HN001 supplemented mice showed higher lymphocyte proliferative responses to PHA than the *S. typhimurium* challenged control (Fig 5). There was no significant difference in the response between mice receiving *B. lactis* HN019 or *L. rhamnosus* HN001 and the uninfected control mice.

Together these results suggest that supplementation with *B. lactis* HN019 or *L. rhamnosus* HN001 is able to confer protection against enteric pathogens such as *Salmonella typhimurium*. Enhanced resistance to infection is accompanied by an increase in immune performance.

CLAIMS:

1. A biologically pure culture of either *L. rhamnosus* HN001 AGAL deposit number NM97/09514 dated 18 August 1997 or *L. rhamnosus* HN067 AGAL deposit number NM97/01925 dated 11 February 1998.

2. A biologically pure culture of *L. rhamnosus* HN001 AGAL deposit number NM97/09514 dated 18 August 1997.

3. A biologically pure culture of *L. rhamnosus* HN067 AGAL deposit number NM97/01925.

4. A composition of a biologically pure culture of any one of *L. rhamnosus* HN001 as claimed in claim 2, *L. rhamnosus* HN067 as claimed in claim 3, *B. lactis* HN019 AGAL deposit number NM97/09513 dated 18 August 1997 or *L. acidophilus* HN017 AGAL deposit number NM97/09515 dated 18 August 1997 in an immunostimulating concentration, with a physiologically acceptable excipient or diluent.

5. A composition as claimed in claim 4 containing any two or more of said strains.

6. A composition as claimed in claim 4 or 5 wherein said physiologically acceptable excipient or diluent is a food.

7. A composition as claimed in claim 6 wherein said food is any one of cultured milk, yoghurt, cheese, milk drink or milk powder.

8. A composition as claimed in claim 4 or 5 which is a pharmaceutical composition and wherein said excipient or diluent is pharmacologically acceptable excipient or diluent.

9. Immunity enhancing physiologically acceptable biologically pure strains of homologues or mutants of any one of the strains:

L. acidophilus HN017,
L. rhamnosus HN001,
B. lactis HN019, or
L. acidophilus HN067.

10. A method of enhancing natural and acquired immunity which comprises administering to a mammal a biologically pure cultures as claimed in any one of claims 1-3 and 9 at an immunostimulating dosage rate.

11. A method as claimed in claim 10 wherein biologically pure cultures of two or three of the above-defined strains are present.

12. A method of enhancing natural and acquired immunity which comprises administering to a mammal a composition as claimed in any one of claims 4 to 8.

13. A method as claimed in claim 10 wherein said physiologically acceptable excipient or diluent is a food.

14. A method as claimed in claim 10 wherein said food is cultured milk, yoghurt, cheese, milk drink or milk powder.

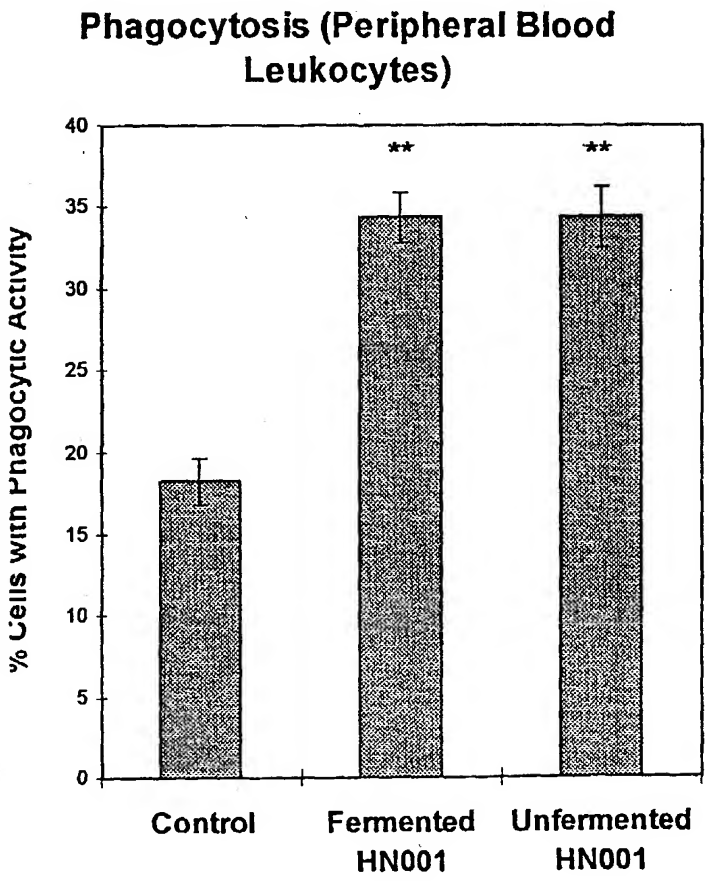
Phagocytosis (Peripheral Blood Leukocytes)
% Cells with Phagocytic Activity
40
35
30
25
20
15
10
5
0
**
**
Control
Fermented HN001
Unfermented HN001

FIG 1

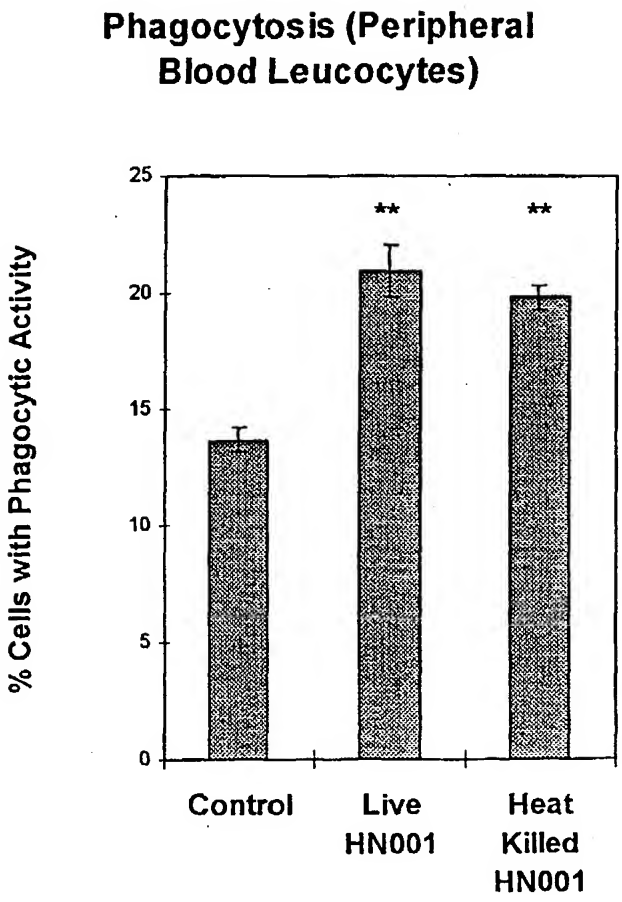
Phagocytosis (Peripheral Blood Leucocytes)
% Cells with Phagocytic Activity
25
20
15
10
5
0
**
**
Control
Live HN001
Heat Killed HN001

FIG 2

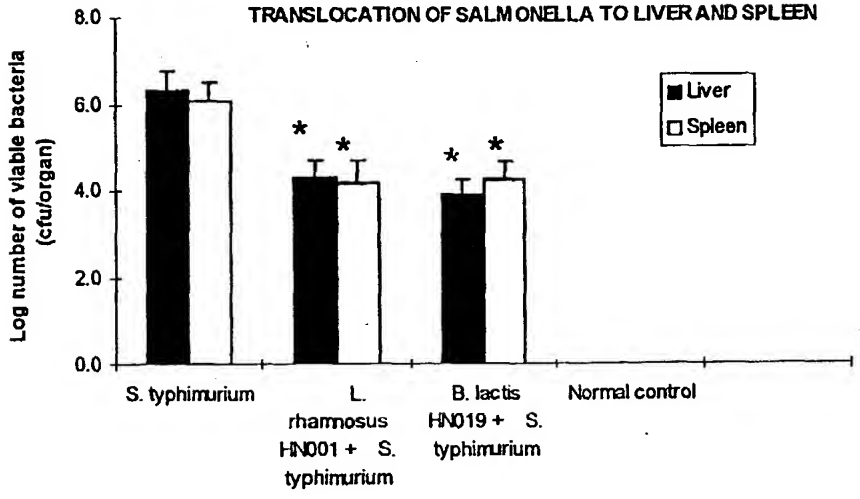
TRANSLOCATION OF SALMONELLA TO LIVER AND SPLEEN
Log number of viable bacteria (cfu/organ)
8.0
6.0
4.0
2.0
0.0
Liver
Spleen
*
*
*
*
S. typhimurium
L. rhamnosus HN001 + S. typhimurium
B. lactis HN019 + S. typhimurium
Normal control

FIG 3

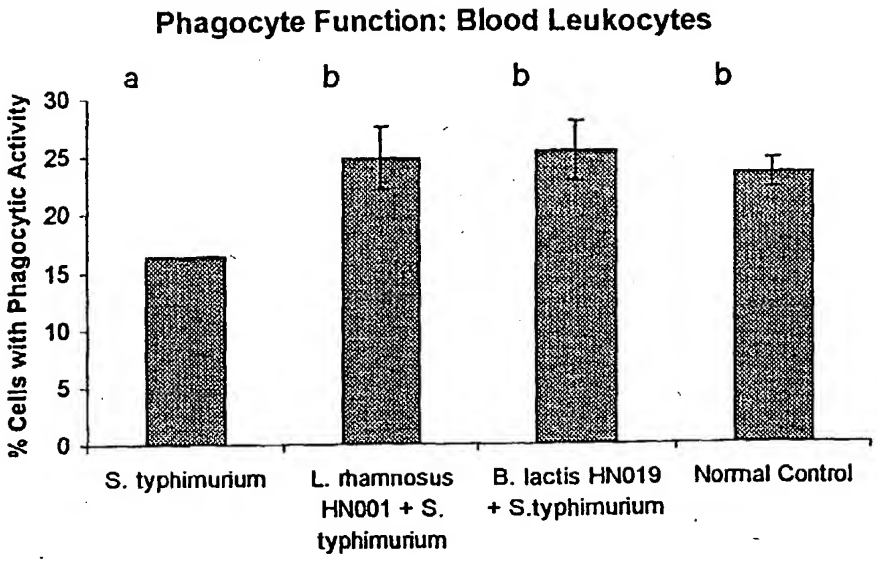
Phagocyte Function: Blood Leukocytes
a
b
b
b
% Cells with Phagocytic Activity
30
25
20
15
10
5
0
S. typhimurium
L. rhamnosus HN001 + S. typhimurium
B. lactis HN019 + S.typhimurium
Normal Control

FIG 4

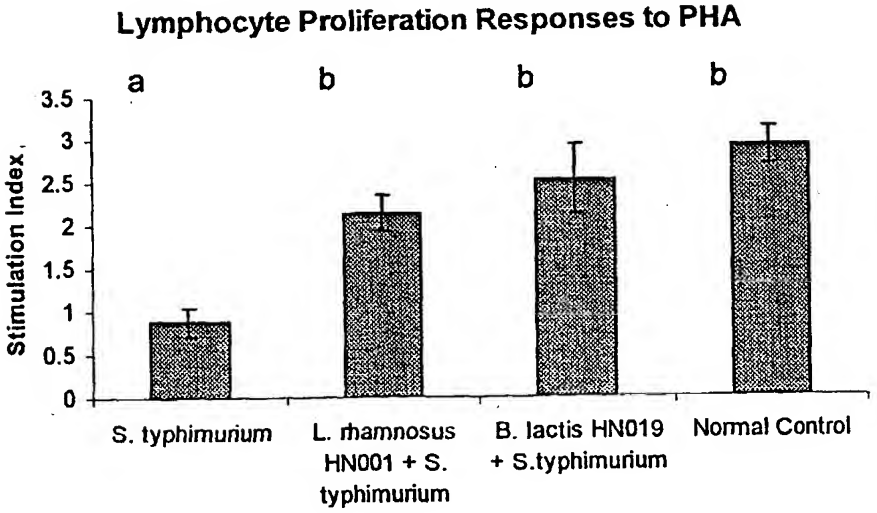
Lymphocyte Proliferation Responses to PHA
a
b
b
b
Stimulation Index
3.5
3
2.5
2
1.5
1
0.5
0
S. typhimurium
L. rhamnosus HN001 + S. typhimurium
B. lactis HN019 + S.typhimurium
Normal Control

FIG 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ 98/00122

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 1/20; A61K 35/74; A23C 9/12, 9/123, 9/127; A23L 1/24; A23C 19/00
// (C12N 1/20; C12R 1:225, 1:23, 1:00)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Electronic Data bases below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Electronic Data bases below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Extra Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0295794 (TOKYO TANABE COMPANY LIMITED) 21 December 1988 whole document	9-14
A	WO 94/18997 (VALIO LTD) 1 September 1994 whole document	1-14
P,A	WO 98/23727 (BIO K^+ INTERNATIONAL INC) 4 June 1998 whole document	1-14

☐ Further documents are listed in the continuation of Box C

☒ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 November 1998	20 NOV 1998

Name and mailing address of the ISA/AU
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ 98/00122

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Each microorganism; *Lactobacillus rhamnosus* HN001, *Lactobacillus rhamnosus* HN067, *Lactobacillus acidophilus* HN017, and *Bifidobacterium lactis* HN019, constitutes a separate invention.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ 98/00122

Box Continuation of Electronic Data Bases

WPAT: 1 Lactobacillus acidophilus HN017 or Lactobacillus rhamnosus HN001 or Lactobacillus rhamnosus HN067 or Bifidobacterium lactis HN019

2 (Lactobacillus or Bifidobacter:) and
(Immun: or lymphocyt: or phagocyt: or tcell or t(w)cell) and
(A61K/IC or A23C/IC)

CA: 1 Lactobacillus acidophilus HN017 or Lactobacillus rhamnosus HN001 or Lactobacillus rhamnosus HN067 or Bifidobacterium lactis HN019

2 (Lactobacillus acidophilus or Lactobacillus rhamnosus or Bifidobacterium lactis) and (immunity or immune or immunogenic)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/NZ 98/00122

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
EP	295794	AU	15519/88	CA	1311204	JP	63291579
		US	5164183				
WO	94/18997	AU	62440/94	CA	2156859	EP	686039
WO	98/23727	AU	51137/98				

END OF ANNEX